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(54) Title: SCREENING, DIAGNOSTIC AND/OR DOSAGE METHOD AND DEVICE OF AN AGONIST AND/OR ANTAGONIST FOR A CALCIUM-COUPLED RECEPTOR

(57) Abstract: The present invention relates to a screening, diagnostic and/or dosage method of a known or unknown agonist (full agonist or partial agonist) and/or a known or unknown antagonist (neutral antagonist or inverse agonist) for a calcium-coupled receptor, comprising following steps:incubating one or more cells expressing apoaequorin and said calcium-coupled receptor with "coelenterazine i" having the formula as depicted in Figure 3 or a derivative thereof, in order to reconstitute active aequorin by said cells; contacting said cell(s) with the agonist and/or antagonist of said receptor on a solid support, and,measuring light emitted by said cell(s), allowing the screening, diagnosis and/or dosage of said agonist and/or antagonist of said receptor, and, possibly, recovering said agonist or antagonist of said receptor from the reaction mixture. The present invention also relates to a corresponding device allowing the performance of said method and to the agonist and/or antagonist of said calcium-coupled receptor identified by said method and device.

SCREENING, DIAGNOSTIC AND/OR DOSAGE METHOD AND DEVICE OF AN AGONIST AND/OR ANTAGONIST FOR A CALCIUM-COUPLED RECEPTOR

Field of the invention

The present invention is related to a screening, diagnostic and/or dosage method of an agonist and/or an antagonist for a membrane-linked receptor, it is also related to a corresponding device allowing the performance of said method, and it is further related to the agonist and/or antagonist of said calcium-coupled receptor identified by said method and device.

Background of the invention and state of the art

Many G-protein-coupled receptors (GPCR) trigger, upon binding of an agonist, a transient increase in the intracellular calcium (Ca^{2+}) concentration. This increase acts as an internal secondary messenger and is an important modulator of many physiological mechanisms (reviewed by Tsunoda (1993), Santella & Carafoli (1997) and Barritt (1999)). Measurement of intracellular calcium concentration in cells expressing a GPCR can thus be used to monitor the efficacy of activation of a GPCR by various compounds known - or suspected - to be ligands for this GPCR.

Changes in calcium concentration can be detected by several means and methods, such as the use of fluorescent dyes (for example: fura-2, fluo-3 and indo-1).

However, calcium sensitive dyes have limitations. Activation of the dyes with an excitation beam requires complicated and expensive instrumentation and limits the use of plastic labware such as microtiter plates.

Another method for intracellular calcium concentration measurement is the use of cell lines

overexpressing a GPCR and apoaequorin, such as described by Sheu et al (1993). In this system, cells expressing apoaequorin are incubated with coelenterazine, which is the co-factor of aequorin. During this incubation, coelenterazine enters the cell and conjugates with apoaequorin to form aequorin, which is the active form of the enzyme. Upon incubation of the cells with an agonist of the GPCR, intracellular calcium concentration increases. This increase leads to the activation of the catalytic activity of aequorin, which oxidises coelenterazine and yields apoaequorin, coelenteramide, CO_2 and light. Once the photon has been emitted, the complex must dissociate and apoaequorin must recombine with a new coelenterazine molecule to be able to emit light again. Thus, in this system, measurement of light emission following agonist addition reflects the ability of said agonist to activate the GPCR and thus to increase intracellular calcium concentration.

However, the state-of-the art use of aequorin for calcium measurement has limitations. In the classical aequorin assay, light is emitted only during 20 to 30 seconds after activation of the GPCR. Thus the emitted light has to be recorded during the few seconds following agonist addition to the cells. This flash-type signal is due to the fact that (1) the intracellular calcium increase triggered by GPCR is only transient, (2) as mentioned earlier, after oxidation of coelenterazine, apoaequorin must recombine with coelenterazine to be able to emit light again and (3) when aequorin is reconstituted with native coelenterazine or classical coelenterazine derivatives, it has a very rapid response to the calcium increase and emission of light follows the profile of the calcium wave.

Thus measurement of an intracellular calcium increase at a high throughput requires complicated and expensive instruments; light measuring instrumentation is required to have built-in injectors so that light can be recorded directly after an addition. The requirement to measure light intensities excludes the use of certain plastic ware such as ordinary microtiter plates, limiting the use to only plates with a clear bottom, which allow simultaneous injection (from the top of the plate) and measurement (from the bottom of the plate). Such plates are more expensive than those plates made only of one sort of plastic.

The EP Patent Application No. 0341477 teaches the expression of the jellyfish photoprotein aequorin in a mammalian cell system, by cloning gene pAQ440 specifying the biosynthesis of the aequorin into an expression vector plasmid of a mammalian cell system, and by subjecting the resulting plasmid to transfection and producing the photoprotein aequorin in the mammalian cell.

The US Patent No. 5,422,266 describes a gene encoding apoaequorin protein included in a vector capable of expressing the apoaequorin in a micro-organism such as *E. coli*.

The US Patent No. 5,714,666 describes mammalian cell lines or transgenic animals expressing apoaequorin and a receptor involved in the modulation of intracellular calcium. This document also describes a method of measuring intracellular calcium comprising adding coelenterazine cofactors to said mammalian cells expressing apoaequorin and measuring photoemission where emission of photons is indicative of intracellular calcium concentration.

PCT Patent Application No. WO 00/02045 teaches a method in which agonists and/or antagonists for

calcium-coupled receptor can be screened for using luminometers equipped with one or six injectors. However, the maximum throughput that can be attained with said method and conventional luminometers can only be considered as "medium throughput" by today's state of the art (i.e. 10 000 data points/day).

Shimomura et al (1989) described the production of synthetic coelenterazine and reconstituted aequorin therewith *in vitro*. Thirty of these semi-synthetic aequorins have the capacity to emit a significant amount of light in the presence of calcium. The relative intensity of the calcium-triggered luminescence of said photoproteins ranged from 0.01 to 190 when compared with the natural aequorin. Two of these (nos 27 and 28) were suggested to be superior to e-aequorin in measuring calcium concentration by the ratio method. Other derivatives were shown to have only a very low relative intensity such as derivatives 6, 7, 8 (i), 9, and 23. This relative intensity is calculated through the ratio of the luminescence intensity of the semi-synthetic aequorin to that of the natural aequorin. The "aequorin h" has a high relative intensity and is currently used in cell-based aequorin assays.

However, the methods of the state of the art require firstly the addition of the native coelenterazine cofactor - or of a chemical derivative of coelenterazine that does not confer significantly different kinetic properties to the enzyme - to cells from a mammalian cell line expressing apoaequorin and incubation to reconstitute a functional aequorin, secondly the preparation of the agent affecting a receptor involved in the modulation of intracellular calcium concentration, thirdly the mixing of said cells with said agent, and finally the measurement of the photoemission.

Furthermore, as mentioned above, light is emitted only during 20 to 30 seconds after activation of the GPCR. Therefore, the recording of the emitted light must be performed during the few seconds following agonist addition to the cells.

Therefore, the methods forming the state of the art are not adequate for high-throughput screening detection which use luminometers having no or few built-in injectors. In addition, said prior art methods do not allow the use of microtiter plates for the testing of thousands of compounds.

Aims of the invention

The present invention aims to provide a method and means, for detecting biologically active substances, especially agonists and/or antagonists for calcium-coupled receptors, said methods not presenting the drawbacks of the state of the art.

A main aim of the present invention is to provide such method and means which allow the detection of biologically active substances, preferably suitable for high-throughput analysis, and which could be adapted to microtiter plates without requiring the addition of built-in injectors inside the screening device.

Another aim of the present invention is to provide an easy and non-expensive method which could be easily automated (preferably suitable for high throughput analysis).

Summary of the invention

The present invention relates to a screening, diagnosic and/or dosage method of a biologically active substance, preferentially a known or unknown agonist (full agonist or partial agonist) and/or a known or unknown

antagonist (neutral antagonist or inverse agonist) for a calcium-coupled receptor, comprising following steps:

(a) incubating one or more cells expressing apoaequorin and said calcium-coupled receptor with "coelenterazine i" having the formula as depicted in Figure 3 or a derivative thereof, in order to reconstitute active aequorin by said cells,

(b) contacting said cell(s) with the agonist and/or antagonist of said receptor ,

(c) measuring light emitted by said cell(s) on a solid support, allowing the screening, diagnosis and/or dosage of said agonist and/or antagonist of said receptor, and,

(d) possibly, recovering said agonist or antagonist of said receptor from the reaction mixture of step (b).

An intrinsic activity is the maximal stimulatory response induced by a compound on a receptor in relation to that of a given reference compound. The numerical value of said intrinsic activity can range from maximal response for full agonists to zero for antagonists, the fractional values within this range denote partial agonists. An inverse agonist is a compound which acts on the same receptor as that of the agonist, yet it produces the opposite effect. Said inverse agonists are also called negative antagonists or reverse agonists.

As used herein the term "screening" is the search for a specific compound out of a pool of different compounds.

As used herein the term "diagnostic method" is an analytical method wherein the compound is characterized as being agonist or an antagonist, and preferentially indicating which type of agonist or antagonist said compound is (eg. partial agonist, full

agonist, neutral antagonist or inverse agonist). The dosage method indicates the efficacy by which the compound binds to said receptor, this is preferentially indicated by EC50 or IC50 values.

As used herein the term "said biological active substance" is a substance of natural origin or synthesized *in vitro*.

The present invention allows said methods to be performed in at low cost and potentially at high-throughput, this in contrast to similar prior art methods.

In the present invention, said "calcium-coupled receptor" refers to a receptor which is naturally coupled to a calcium pathway or to a receptor which naturally is not directed to a calcium pathway but is redirected to this particular pathway. It is known by a person skilled in the art that, for example certain G-protein coupled receptors may couple to Gs, Gi, Go, Gq-11 proteins. Receptors coupling naturally to a G-protein belonging to the Gq-11 family results automatically in the modulation of the intracellular calcium pathway. In contrast, other receptors regulate the production of cAMP via Gs and Gi and do naturally not influence the intracellular calcium level directly. In addition, it is known by a person skilled in the art that said latter receptors may couple promiscuously to G16 which leads to the change of intracellular calcium. Consequently, said receptors may move to another intracellular pathway depending on the coupling molecules present in the cell. It is not excluded that receptors other than GPCR may be directed to the calcium pathway.

As previously pointed out, different coelenterazines may be used to reconstitute active aequorin. Till now, only aequorins having a high relative intensity are used in cell based aequorin assays. The

present invention found surprisingly that the semi-synthetic aequorins 6, 7, and 8 (i), which have a very low relative intensity, are superior to aequorin h in the detection of (ant)agonists for calcium-coupled receptors. Furthermore, they are suitable for use in high-throughput and low cost luminometers with low cost assay plates.

The present invention illustrates that, despite their low relative intensities, said semi-synthetic aequorins emit sufficient light to enable quantitative calcium changes to be determined. In addition to their low relative intensity, aequorins 6, 7, and 8 (i) have a high half-total time, which is the time required to emit 50% of the total light compared to the other aequorins in the same assay conditions. Due to the combination of a longer half-total time and the relatively high light-emitting capacity of said compounds (although with low relative intensity) present inventors found surprisingly that said aquorins are superior for measuring intracellular calcium changes using an inexpensive luminometer. The present invention illustrates that aequorin i allows the formation of a delayed signal, permitting the use of low-cost luminometers and low cost assay plates without interfering with the quality and/or sensitivity of the assay as such. Therefore the present invention proposes to use coelentarazine i (or derivatives thereof such as 6 and 7) in order to reconstitute "aequorin i" in assays allowing the performance of high-throughput assays in a low cost mode.

"aequorin" is defined as a natural wild type aequorin or derivative thereof (improved aequorin comprising one or more specific mutations, or homologous proteins of aequorin which are, for instance, described in the document EP-0 341 477, US-5,422,266, US-5,714,66, EP-0 540 064, EP-0 187 519 (US 5,541,309), Stables et al.,

Analytical Biochemistry, Vol.252, p.115-126 (1997) (US 5,360,728).

"coelenterazine i" and derivatives thereof, are defined as the molecule(s) presented in the formula as depicted in Figure 3, as well as its derivatives allowing similarly the formation of a delayed signal, preferably a light emission which could be recorded between about 5 and about 20 seconds or more after mixing of the cells and the biologically active substance(s). Derivatives of said "coelenterazine i" could be similar to coelenterazine, comprising substitution by another halogen (F, Cl, Br) or wherein one or more non-functional groups are substituted by other non-functional groups by methods well-known by the person skilled in the art (see also crystallographic analysis of aequorin and coelenterazine (Head J.F. et al., Nature 405, pp. 372-376 (2000)). Therefore, the present invention also relates to a screening, diagnostic and/or dosage method, wherein the derivative of coelenterazine i, is a compound similar to coelenterazine i as depicted in Figure 3 wherein the I is substituted by another halogen chosen from the group consisting of F, Cl, Br; or wherein one or more non-functional groups of coelenterazine i are substituted by other non-functional groups.

Alternatively, "coelenterazine i" could be presented and expressed by cell(s). According to the present invention, said cells may be adhering cell(s) or cell(s) in suspension. In addition, said cells may be of eukaryotic or prokaryotic origin. For example said eukaryotic cells may be of human, mammalian, animal, plant, fungi, or of yeast origin. Alternatively, said cells may be bacterial cells.

According to the present invention, the method which is used to perform the screening, diagnostic and/or dosage method may vary depending on the compound

being analysed. Certain manipulations are not mentioned in the listing of the performed steps of the methods of the present invention as they may be logically deduced from the description itself. For example, the solid support is kept outside the luminometer when not transferred to the luminometer, or the mixing is performed within the lunimometer when the solid support is already transferred to the luminometer.

For example, the screening, diagnostic and/or dosage method of a known or unknown agonist or a known or unknown inverse agonist for a calcium-coupled receptor according to the present invention may comprise the following successive steps:

(a) contacting the agonist or the inverse agonist (tested substance) with a solid support,

(b) incubating one or more cell(s) expressing apoaequorin and said calcium-coupled receptor with coelenterazine i as depicted in Figure 3 or a derivative thereof in order to reconstitute active aequorin by said cell(s),

(c) mixing said agonist or inverse agonist with said cell(s) on said solid support,

OR

transferring said solid support to a luminometer and mixing said agonist or inverse agonist with said cells in said luminometer, said luminometer being equipped with built-in injector(s),

(d) optionally incubating said cells with said tested substance,

(e) optionally transferring said solid support to a luminometer when said solid support was not previously inserted in the luminometer in step (c),

(f) obtaining a measurement of light emitted by said cell(s),

(g) determining from the results of said measurement whether the tested substance is an agonist or an inverse agonist of said receptor, and, possibly determining the affinity of said tested substance for said receptor, and,

(h) possibly recovering said agonist or said inverse agonist of said receptor from the reaction mixture of step (d).

In this experimental set up, when the method is applied to screen, diagnose and/or dose an inverse agonist of the calcium-coupled receptor, said receptor preferentially possesses a constitutive activity. This means that the receptor is continuously activated in the cells at rest (not activated by an agonist) resulting in the presence of an increased basal intracellular calcium level. In the method of the present invention this basal calcium level may consume the activated aequorin resulting in the production of basal levels of light. Inverse agonists can be easily identified through the detection of the lowering of said basal level upon treatment of said cells with said inverse agonist.

In order to evaluate whether an agonist is a full or a partial agonist, a reference molecule is used in the experiment (preferentially a full agonist) allowing the comparison of the intensity of the emitted light for both compounds when incubated with the cells of the present invention.

Alternatively, the screening, diagnostic and/or dosage method for a known or an unknown antagonist (neutral antagonist or inverse agonist) according to the present invention may comprise the following successive steps:

(a) contacting the antagonist (tested substance) with a solid support,

(b) incubating one or more cell(s) expressing apoaequorin and said calcium-coupled receptor with coelenterazine i as depicted in Figure 3 or a derivative thereof in order to reconstitute active aequorin by said cell(s),

(c) mixing said antagonist (tested substance) with said cell(s) on said solid support,

(d) optionally incubating said cells with said antagonist,

(e) optionally transferring said solid support to a luminometer and obtaining a measurement of light emitted by said cell(s),

(f) adding a known agonist of the receptor to the mixed cell(s) and tested substance to determine whether the tested substance is an antagonist of the receptor,

(g) optionally transferring said solid support to a luminometer and possibly obtaining a second measurement of light emitted by said cell(s),

(h) determining from the results of steps (e) and/or (g) whether the tested substance is an antagonist of said receptor, and, possibly determining the affinity of said tested substance for said receptor, and,

(i) possibly recovering said antagonist of said receptor from the reaction mixture of step (f).

When the cells are preincubated with an antagonist, before being treated with an agonist of the same receptor, said receptor may become occupied by the antagonist before the agonist has the possibility to bind and activate the receptor. The activity of said antagonist is thus studied by their capacity for blocking the agonist activation of said receptor. In the method of the present invention said inhibition is reflected by the inhibition of the light emission.

In addition, the screening, diagnostic and/or dosage method for a known or an unknown antagonist (neutral antagonist or inverse agonist) according to the present invention may comprise the following successive steps:

(a) contacting a known agonist of said calcium-coupled receptor with a solid support,

(b) incubating one or more cell(s) expressing apoaequorin and said calcium-coupled receptor with coelenterazine i as depicted in Figure 3 or a derivative thereof in order to reconstitute active aequorin by said cell(s),

(c) mixing said antagonist (tested substance) with said cell(s) in a vial other than the solid support,

(d) optionally incubating said cells with said antagonist,

(e) optionally transferring said device to a luminometer and obtaining a measurement of light emitted by said cell(s),

(f) adding said cell(s) to the agonist on the solid support to determine whether the tested substance is an antagonist of the receptor,

(g) optionally transferring said solid support to a luminometer and possibly obtaining a second measurement of light emitted by said cell(s),

(h) determining from the results of steps (e) and/or (g) whether the tested substance is an antagonist of said receptor, and, possibly determining the affinity of said tested substance for said receptor, and,

(i) possibly recovering said antagonist of said receptor from the reaction mixture of step (f).

Alternatively, the screening, diagnostic and/or dosage method for a known or unknown agonist or a

known or unknown inverse agonist according to the present invention may comprise the following successive steps:

(a) incubating one or more cell(s) expressing apoaequorin and said calcium-coupled receptor with coelenterazine i as depicted in Figure 3 or a derivative thereof in order to reconstitute active aequorin by said cell(s),

(b) mixing said agonist or inverse agonist (tested substance) with the cell(s) of step (a) in a vial other than the solid support,

(c) adding the mixture obtained in step (b) on a solid support,

(d) optionally incubating said cells with said tested substance,

(e) optionally transferring said solid support to a luminometer and obtaining a measurement of light emitted by said cell(s),

(f) determining from the results of said measurement whether the tested substance is an agonist (full or partial) or an inverse agonist of said receptor, and, possibly determining the affinity of said tested substance for said receptor, and,

(g) possibly recovering said agonist or said inverse agonist of said receptor from the reaction mixture of step (d).

Also in this experimental set up, when the method is applied to screen, diagnose and/or dose an inverse agonist of the calcium-coupled receptor, said receptor preferentially possesses a constitutive activity.

In addition, the screening, diagnostic and/or dosage method of a known or an unknown agonist (full or partial) or a known or unknown inverse agonist according to the present invention may comprise the following successive steps:

(a) contacting one or more cells with a solid support, said cell(s) expressing apoaequorin and said calcium-coupled receptor, incubating said cells with coelenterazine i as depicted in Figure 3 or a derivative thereof in order to reconstitute active aequorin by said cell(s),

(b) adding to said solid support the agonist or the inverse agonist (tested substance) for said calcium-coupled receptor,

OR,

transferring said solid support to a luminometer and mixing said agonist or inverse agonist with said cells in said luminometer, said luminometer being equipped with built-in injector(s),

(c) optionally incubating said cells with said tested substance,

(d) optionally transferring said solid support to a luminometer when said solid support was not previously inserted in the luminometer in step (b),

(e) obtaining a measurement of light emitted by said cell(s),

(f) determining from the results of said measurement whether the tested substance is an agonist or an inverse agonist of said receptor, and, possibly determining the affinity of said tested substance for said receptor, and,

(g) possibly recovering said agonist or said inverse agonist of said receptor from the reaction mixture of step (c).

Additionally, the screening, diagnostic and/or dosage method of known or an unknown antagonists (neutral antagonist or inverse agonist) for a calcium-coupled receptor according to the present invention may comprising the following successive steps:

(a) contacting one or more cells with a solid support, said cell(s) expressing apoaequorin and said calcium-coupled receptor, incubating said cells with coelenterazine i as depicted in Figure 3 or a derivative thereof in order to reconstitute active aequorin by said cell(s),

(b) adding to said solid support the antagonist (tested substance) for said calcium-coupled receptor,

OR,

transferring said solid support to a luminometer and mixing said antagonist with said cells in said luminometer, said luminometer being equipped with built-in injector(s), and obtaining a measurement of an emitted light by said cell(s),

(c) optionally incubating said cells with said antagonist,

(d) possibly transferring said solid support at step (c) to a luminometer and obtaining a measurement of light emitted by said cell(s),

OR

keeping said solid support outside the luminometer,

(e) adding a known agonist of the receptor to the mixed cell(s) and the tested substance to determine if the tested substance is an antagonist of the receptor,

(f) optionally moving said solid support in a luminometer if not inserted at step (d)

(g) possibly obtaining a second measurement of an emitted light by said cell(s),

(h) determining from the results of step (d) and/or (g) whether the tested substance is an antagonist of said receptor, and, possibly determining the affinity of said tested substance for said receptor, and,

(i) possibly recovering said antagonist of said receptor from the reaction mixture of step (e).

Alternatively, the screening, diagnostic and/or dosage method of a known or an unknown antagonist (neutral antagonist or inverse agonist) for a calcium-coupled receptor according to the present invention, comprising the following successive steps:

(a) preparing one or more cells in a separate vial, said cell(s) expressing apoaequorin and said calcium-coupled receptor, incubating said cells with coelenterazine i as depicted in Figure 3 or a derivative thereof in order to reconstitute active aequorin by said cell(s),

(b) adding to said cells the antagonist (tested substance) for said calcium-coupled receptor,

(c) transferring the cells of step (b) to a solid support,

(d) optionally incubating said cells with said antagonist before or after the transfer of said mixture to said solid support,

(e) possibly transferring said solid support to a luminometer, and obtaining a measurement of light emitted by said cell(s),
OR,
keeping the solid support outside the luminometer

(f) adding an agonist of the receptor to the mixed cell(s) and the tested substance to determine if the tested substance is an antagonist of the receptor,

(g) transferring said solid support to a luminometer if not inserted at step (e) and possibly obtaining a second measurement of light emitted by said cell(s),

(h) determining from the results of steps (e) and/or (g) whether the tested substance is an antagonist of said receptor, and, possibly determining the affinity of said tested substance for said receptor, and,

(i) possibly recovering said antaagonist of said receptor from the reaction mixture of step (f).

Similarly, the screening, diagnostic and/or dosage method for a known or a unknown antagonist (neutral antagonist or inverse agonist) according to the present invention may comprise the following successive steps:

(a) contacting said antagonist (tested substance) with a known agonist for said receptor with a solid support,

(b) incubating one or more cell(s) expressing apoaequorin and said calcium-coupled receptor with coelenterazine i as depicted in Figure 3 or a derivative thereof in order to reconstitute active aequorin by said cell(s),

(c) mixing said antagonist/agonist mixture with said cell(s) on said solid support,

(d) optionally incubating said cells with said tested substance,

(e) transferring said solid support to a luminometer and obtaining a measurement of light emitted by said cell(s),

(f) determining from the results of said measurement whether the tested substance is an antagonist of said receptor, and,

(g) possibly recovering said antagonist of said receptor from the reaction mixture of step (d).

In this experimental set up, an agonist and an antagonist for the same receptor is added at the same time to the cells expressing said receptor. If in this case the agonist

binds before the antagonist onto this receptor, the receptor may become activated resulting in the emission of light. Depending on the binding kinetics of the compound onto said receptor, said agonist may dissociate from said receptor and be replaced by the antagonist. In this case, antagonistic properties may be detected using the method according to the present invention.

Similarly, the screening, diagnostic and/or dosage method of a known or an unknown antagonist (neutral antagonist or an inverse agonist) according to the present invention may comprise the following successive steps:

(a) contacting one or more cells with a solid support, said cell(s) expressing apoaequorin and said calcium-coupled receptor, incubating said cells with coelenterazine i as depicted in Figure 3 or a derivative thereof in order to reconstitute active aequorin by said cell(s),

(b) adding to said solid support a known agonist and the tested substance which is an antagonist (neutral antagonist or an inverse agonist) for said calcium-coupled receptor,

(c) optionally incubating said cells with said antagonist/agonist mixture,

(d) transferring said solid support to a luminometer,

(e) obtaining a measurement of light emitted by said cell(s),

(f) determining from the results of said measurement whether the tested substance is an antagonist of said receptor, and, possibly determining the affinity of said tested substance for said receptor, and,

(g) possibly recovering said antagonist of said receptor from the reaction mixture of step (c).

More particularly, the screening, diagnostic and/or dosage method according to the present invention, characterised in that the cell expresses apoaequorin in the cytoplasm or in the mitochondria or in any other part of the cell.

According to the present invention, said cell expressing the calcium-coupled receptor may also expresses proteins (endogenous or overexpressed) ensuring a coupling of said receptor (endogenous or overexpressed) to a calcium pathway. According to the present invention, said protein may be selected from the group consisting of natural Gα16 protein, chimeric G-protein resulting from a fusion between two different G-proteins or phospholipase Cβ2 protein or any other coupling protein or chemical.

More in particular, the calcium-coupled receptor of the present invention may be a G-protein-coupled receptor (GPCR).

According to the present invention, the measurement of the emitted light is obtained with one or more luminometer(s) equipped with several dispensers and measurement heads or with one or more luminometer(s) wherein no built-in dispensers are present.)

Preferentially, said luminometer may be chosen from the group consisting of the NorthStar (PE-Biosystems) Microlumat (Berthold), FLIPR (Molecular Devices) and ImageTrack (Packard).

In addition, according to the present invention, the solid support is a microtiter plate.

Advantageously, the solid support is a microtiter plate, preferably a 96-wells, 384-wells, a 1536 wells microtiter plate or smaller format.

Preferably, the present invention relates to a high throughput screening, diagnostic and/or dosage method.

The present invention is also related to a (preferably high-throughput) screening, diagnostic and/or dosage device intended for the screening method according to the invention, said device comprising the following elements :

- a microtiter plate, preferably a 96-well or 384-well microtiter plate,
- a medium containing cell(s) expressing apoaequorin and a calcium-coupled receptor,
- a medium containing said coelenterazine i or a derivative thereof, and
- means (such as one or more luminometer(s) equipped with one or several measurement heads and placed beside one or several dispensers) for detecting an emitted light by said cell(s).

Advantageously, the device and dispensers according to the invention comprise means for automatically performing the successive steps of the screening, diagnostic and/or dosage method according to the invention.

In addition, the present invention also relates to a kit for high throughput screening, diagnostic and/or dosage method of a known or unknown agonist and/or a known or an unknown antagonist of a calcium-coupled receptor using a luminometer with a several dispensers and measurement heads or using a luminometer without built-in dispensers; comprising coelentrazine i as depicted in Figure 3 or a derivative thereof.

The present invention also relates to unknown agonist or unknown antagonist of a receptor identified by the method according to a method of the present invention.

More in particular, the present invention relates to the use of an unknown agonist and/or an unknown antagonist of a receptor in the preparation of a medicament for treating diseases influenced by said receptor.

Said disease may be any disease influenced by a receptor, for example this disease may be a mood-related disease, mental-related disorders or a gastrointestinal-related disease. Other diseases influenced by receptors are known by a person skilled in the art.

The present invention also involves the use of a method according to the present invention for the screening, diagnosis and/or dosage of a known or unknown agonist (full agonist or partial agonist) and/or a known or unknown antagonist (neutral antagonist or inverse agonist) for a membrane-linked receptor. Said membrane-linked receptor may be a G-protein-coupled receptor.

A last aspect of the present invention is related to a method to screen for, to diagnose and/or dose a known or unknown agonist (full agonist or partial agonist) and/or a known or unknown antagonist (neutral antagonist or inverse agonist) for a membrane-linked receptor capable of modifying the cellular metabolism in a subject. Said subject may be of human, animal or plant origin.

A last aspect of the invention is related to the unknown agonist and/or antagonist of a calcium-coupled receptor identified by the method and device according to the invention.

The present invention will be described in details in the following non-limiting examples, in reference to the enclosed Figures.

Brief description of the drawings

Figure 1 describes the general principle of reconstituting an active aequorin from the protein apoaequorin expressed from a plasmid transfected in a cell. The coding region of said aequorin may also be present chromosomally. The coelenterazine may be natural or made synthetically. This figure also presents the principle of calcium-dependent generation of light by aequorin by the oxidation of coelenterazine by the enzyme.

Figure 2 describes the principle of the cell-based aequorin assay, as in the method of the present invention. In this illustration, the receptor is a GPCR, but it could also be any other type of receptor, such as ion-channels or tyrosine kinase receptors.

Figure 3 presents the structure of the natural substrate of the enzyme (Native coelenterazine), of a derivative obtained by chemical synthesis that is currently used in cell-based aequorin assays (coelenterazine h) and of coelenterazine i as used in the method according to the invention.

Figure 4 presents the enzymatic properties of aequorin when it is reconstituted with different kinds of synthetic coelenterazines in an *in vitro* aequorin assay, as disclosed in the literature (Shimomura *et al.*, 1989, Shimomura, 1991).

Figure 5 gives the intensity of the emitted light (in Relative Light Units (RLU)) recorded for 30 seconds after injecting a cell suspension into wells of a microtiter plate, said cell suspension comprising cells co-expressing apoaequorin and the serotonin receptor 5HT2B and being incubated with coelenterazine h (upper panel) or coelenterazine i (lower panel), each well containing initially either digitonin (marked as "Digi") or serotonin (upper and lower panels).

Figure 6 is a graphical analysis of the delay in the luminescent signal obtained with coelenterazine i, in comparison with some other kinds of coelenterazines (h, fcp and ip) in cell-based aequorin assays. Cells were treated as described in Figure 5 and their response to Digitonin (open symbols) or their response to serotonin (5HT, closed symbols)- an agonist of a receptor expressed by the cells- was analysed. The time at which the maximum emission of light occured was calculated with the « Winglow » software provided with the « microlumat » luminometer (Berthold) and is represented here as a function of the concentration of serotonin (left part of the figure) or for a saturating concentration of digitonin (10 µM, right part of the figure).

Figure 7 gives the dose-response curve for serotonin on the 5HT-2B receptor in cell-based aequorin assays using either coelenterazine h or coelenterazine i, as obtained by the same analysis procedure as in Figure 6. The curve gives the intensity of light emitted in RLU as a function of the logarithm of the final concentration of the serotonin, the integration of emitted light being calculated for 30 seconds for left panel or for 22 seconds for right panel.

Figure 8 represents the results obtained with the same cell-based aequorin assay and analysis as described in Figure 7, but here cells expressed a histamine H1 receptor instead of a 5HT-2B receptor, and histamine was used as an agonist of this receptor.

Figure 9 represents the results obtained with the same cell-based aequorin assay as described in Figures 7 and 8, but here cells expressed the orexin receptor 2 instead of a serotonin receptor, and orexin B was used as an agonist of this receptor.

Figure 10 gives the dose-response curve for MCH on the MCH receptor in cell-based aequorin assays using a luminometer where there is a delay between the last injection and the beginning of the recording of the emitted light. Values (RLU, Relative Luminescence Units) are plotted against the logarithm of the agonist concentration to yield the sigmoidal dose-response curves. The half maximal agonist concentration (EC50) and the maximal effect (TOP) were calculated from these curves. Digitonin was used as a control of the aequorin content of the cells. Several curves, corresponding to the signal recorded on different columns of the assay plate, are shown.

Figure 11 gives the dose-response curve for serotonin on the 5HT-2B receptor preincubated with the antagonist mesulergine in cell-based aequorin assays using either coelenterazine h or coelenterazine i. Values (RLU, Relative Luminescence Units) were plotted against the logarithm of the antagonist concentration to yield the sigmoidal dose-response curves shown. Half maximal agonist concentration (IC50) was calculated from these curves. Digitonin was used as a control of the aequorin content of the cells.

Description of a preferred embodiment of the present invention

The method according to the invention is related to the detection of agonistic or antagonistic activities of substances of membrane-linked receptors by means of mammalian cells lines expressing apoaequorin and a said receptor (eg. GPCR).

Figure 1 summarises the general principle of the calcium-dependent generation of light by aequorin following oxidation of coelenterazine, as used in the

method of the present invention, while Figure 2 explains how, in said method, the binding of an agonist to a GPCR leads, through molecular signalling, to light emission.

Figure 3 gives the chemical structure of coelenterazine i. The synthesis and chemical structure of said coelenterazine i have been previously described by Shimomura *et al.* (1989). This structure is compared to the structure of the natural substrate of the enzyme (Native coelenterazine) and to the structure of a derivative obtained by chemical synthesis, which is currently used in cell-based aequorin assays and which is called "coelenterazine h". The circles indicate the differences of composition between these 3 molecules. In coelenterazine i, the hydroxyl group of native coelenterazine (-OH) has been replaced by an iodine atom (-I).

The enzymatic properties of aequorin, when it is reconstituted with different kinds of synthetic coelenterazines, have been described in the literature (Shimomura et al., 1989, Shimomura, 1991) and are presented in Figure 4. These enzymatic properties correspond to properties for an "in vitro" aequorin reaction - not for aequorin used in cellular assay. The use of coelenterazine i for an aequorin-based cellular assay has never been described in the literature.

Detection of agonistic or antagonistic activities of substances of membrane-linked receptors by means of mammalian cell lines expressing apoaequorin and a calcium-coupled receptor (eg. GPCR) requires the measurement of emitted light to be performed just after placing the cells -pretreated with or without an antagonist- in contact with the supposed agonist. This emitted light can easily be measured at low throughput using a single-tube luminometer. However, up to now, this

biological system could not be used at a high-throughput scale. Indeed :

(1) the necessity to measure light just after placing the cells in contact with the agonist to be tested requires the use of a luminometer equipped with a built-in dispenser. For example, due to the short duration of light emission, it is impossible to inject the drugs to be tested on the cells placed in the 96 wells while the plate is outside the luminometer, to subsequently transfer the plate to the luminometer and record emitted light. Even if the plate could rapidly (i.e. in less than 15 seconds) be placed in the luminometer after injection of the drugs to be tested, current apparatuses do not allow the measurement of light from the 96 wells before the extinction of the flash signal of aequorin, as these luminometers are not equipped with 96 detectors.

(2) Luminometers able to inject simultaneously a different compound in each of the wells of a 96 or 384-well plate and start immediately the measurement of the luminescent signal are just becoming to be available on the market (for example the FDSS, made by Hamamatsu Photonics, Japan), but these devices are expensive and require the use of expensive clear-bottom plates. Other luminometers are able to read all the 96 or 384-well plate at once, but are not equipped with built-in dispensers (for example the CLIPR, made by Molecular Devices; the NorthStar, made by PE-Biosystems; ImageTrack, made by Packard). Up to now, these luminometers could not be used for the measurement of cell-based aequorin assay, due to the short duration of the luminescent signal.

(3) Another invention made by the authors of the present invention (PCT Patent Application No. WO 00/02045) teaches how it is possible to increase the throughput of luminometers equipped with one or six injectors. However, the throughput that can be reached with this method and conventional luminometers is only considered as "medium throughput" today (i.e. 10 000 data points/day).

The present invention provides a method for performing high-throughput screening of drugs binding to a calcium-coupled receptor (eg. GPCR) by the use of mammalian cell lines expressing apoaequorin and a calcium-coupled receptor (eg. GPCR) and by the use of a luminometer that is equipped with no or few built-in dispensers. Following this method, the solutions to be tested for (ant)agonistic activities are placed in the wells of a 96-well plate. Cells expressing apoaequorin and a calcium-coupled receptor (eg. GPCR) may be detached from the culture plate and are incubated with coelenterazine i to reconstitute active aequorin. Alternatively, said cells may be cultured in suspension, if they are suitable for use in such conditions.

After incubation with coelenterazine i, these cells are then maintained in suspension with a magnetic stirrer and the cell suspension is injected into the solutions of supposed agonist to be tested.

The plate is then transferred to the luminometer, at the position where measurement of the emitted light can occur. Light emission is then recorded for 1 (alternatively up to 60) second(s). This method delays the emission of light by aequorin present in the cell and allows up to 384 measurements of agonist-induced aequorin light emission to be taken in 1 minute or less, using a luminometer having few or no built-in dispenser

(for example with the CLIPR or the NorthStar), when said dispensers is located at a position which is not the reading position (for example placed beside the luminometer).

This method thus allows the high-throughput screening (>100 000 samples/day) of mammalian cell lines expressing apoaequorin and a calcium-coupled receptor (eg. GPCR) by the use of a luminometer devoid of built-in dispenser. This opens a new field of use for this kind of luminometer.

This system also facilitates a functional screening using very few (down to 100) cells per measurement, due to the high sensitivity of the CCD-cameras present in this kind of luminometer.

The method according to the invention is suitable for performing high-throughput analysis of calcium-coupled receptor (eg. GPCR) stimulation by known or supposed agonists or antagonist by means of cells expressing the receptor and apoaequorin. These cells may express apoaequorin in the cytoplasm, as described by Sheu et al. (1993) or Button and Brownstein (1993) or may express apoaequorin in the mitochondria, by means of the addition of a mitochondrial targeting sequence to the aequorin, as used by Stables et al. (1997) or in any other part of the cell. These cells may also express proteins intended to ensure coupling of the over-expressed receptor to the calcium pathway. These may be the natural Gα16 protein (Milligan et al., 1996) or its murine counterpart Gα15, chimeric G proteins resulting from a fusion between two different G proteins (Komatsuzaki et al., 1997), phospholipase C-β2 (Park et al., 1992), or any other "universal coupling" protein.

Examples

Example 1

In this example, reference is made to the results presented in Figure 5.

Cells co-expressing apoaequorin and the serotonin receptor 5HT2B were detached from the culture plates and aequorin was reconstituted by incubating the cells in suspension with 5 µM coelenterazine h (upper panel of Figure 5) or with 5 µM coelenterazine i (lower panel of Figure 5). Cell suspension was then injected either into digitonin (marked as "Digi" on Figure 5), said digitonin allowing the entry of extracellular calcium into the cells, or into dilutions of serotonin (5HT), said serotonin acting on the 5HT2B receptor expressed by the cells to increase cytoplasmic and mitochondrial calcium concentration via the release of intracelular calcium stores. Emission of light was recorded for 30 seconds for each well immediately after injection of the cell suspension. It should be noted that, in these experimental results, the intensity of the emitted light is expressed in Relative Light Units (RLU), as a function of time.

More precisely, a CHO cell line expressing the serotonin 5HT2B receptor, the Gα16 coupling protein and apoaequorin was established. Cells were cultivated as a monolayer in HAM'sF12 medium containing 10% Foetal bovine serum (FBS). On the day of the experiment, the culture medium was removed and cells were incubated for 5 min at room temperature in PBS-EDTA (phosphate buffered saline solution without calcium, added with 5 mM EDTA). Cells were detached from the culture vessel by shaking the culture plate by hand and by pipetting up and down. Cells were centrifuged and the supernatant was removed to eliminate the EDTA; the pellet was resuspended in culture medium

DMEM:HAM'sF12 with Hepes, without FBS and with 0.1% Bovine Serum Albumin. Cells were counted by means of a Thomas cell, were centrifuged again and were resuspended in culture medium DMEM:HAM'sF12 with Hepes, without FBS and with 0.1% Bovine Serum Albumin at a concentration of 5.10^6 cells/ml. Coelenterazine i (obtained from Biotium Inc., http://biotium.com) at 500 µM in methanol was added to the cell suspension at a final concentration of 5 µM. The cell suspension was then stored in the dark at room temperature for 3 to 5 h, with continuous shaking by magnetic stirring to maintain the cells in suspension.

A series of dilutions of serotonin, a known ligand of the 5HT2B receptor, was prepared in culture medium DMEM:HAM'sF12 with Hepes (without FBS and with 0.1% Bovine Serum Albumin) and 50 µl of each of these solutions were placed in the wells of a 96-well plate. The cell suspension was diluted 10 times with medium HAM'sF12 (without FBS and with 0.1% Bovine Serum Albumin) and was placed in a glass container protected from light by wrapping it with aluminium paper. A magnetic stirring bar was added to the suspension and a magnetic stirrer was used at low speed (1 to 5 rounds per second) to maintain the cells in an homogenous suspension. The magnetic stirring bar was equipped with a ring to reduce the possibility of fracturing the cells and to reduce the subsequently release of aequorin in the culture medium. Alternatively, a culture vessel equipped for culture of cells in suspension may be used.

The use of the EG&G Wallac's MicroLumat-Plus microplate luminometer, allows injection and direct subsequent recording of the light emissions from each well of a 96-well plate with time, enabling kinetic measurements to be made. The end of the entrance tube of the dispenser

was placed at the bottom of the cell suspension and the dispenser was filled with a volume of suspension 3 times the dead volume of the apparatus so that the volume occupied by the tube and pumps were completely filled with cell suspension. The 96-well plate containing the solutions of agonists was then inserted into the luminometer. Then, for each well, 50 µl of the cell suspension (i.e. 25 000 cells) was dispensed into the well and the emitted light was immediately recorded during 30 seconds. After reading the first well, cells were injected into the next well and emitted light was recorded, etc. For each plate, a series of curves representing the intensity of the emitted light as a function of time for each well was displayed.

The same experiment was made by incubating the cell suspension from the CHO cell line with coelenterazine h instead of coelenterazine i, to a final concentration of 5 µM. Results are presented on Figure 5, upper panel.

Moreover, comparison was also made by replacing the addition of serotonin dilutions to cell suspension with the addition of digitonin dilutions (marked as "Digi" on Figure 5).

Results of Figure 5 show that the use of aequorin reconstituted with coëlenterazine i allows the delay of the luminescent response of aequorin to the intracellular calcium concentration increase.

On the basis of the results according to Figure 5, the intensity of the emitted light was integrated over 30 seconds, or from second 8 to second 30 using the Winglow software provided with the luminometer, yielding, for each well, one value representative of the emitted light and hence of the stimulation of the 5HT2B receptor by the agonist present in the well. These values can be

plotted against the logarithm of the ligand concentration to generate dose-response curve as shown in Figure 7. These allow the determination of half-maximal response doses (EC_{50}) for each ligand.

More precisely, Figure 7 represents the dose-response curve for serotonin on the 5HT-2B receptor which represent RLU (integration of emitted light for 30 seconds for left panel or for 22 seconds for right panel) according to the logarithm of the final concentration of the serotonin. This is another graphical analysis of the delay in the luminescent signal obtained with coelenterazine i, in comparison with coelenterazine h. Cells were treated as described in Figure 5 and their kinetic of response to serotonin (5HT), an agonist of the 5HT-2B receptor expressed by the cells, were recorded with the Microlumat luminometer and the software « Winglow » (as shown in Figure 5). On the left panel, the emitted light was integrated from the time of contact between the cells and the agonist of the receptor (t = 0 s) until the end of the measurement (t = 30 s), yielding a single value for each data point. The said integrated measurement was then plotted against the logarithm of the concentration of the agonist, to yield a typical sigmoidal dose-response curve for cells loaded with coelenterazine h or with coelenterazine i. On the right panel, the light emitted during the first 8 seconds after the mixing was omitted, and integration of the signal was performed between second 8 and second 30. This corresponds to the part of the signal that could be read by a device where mixing of the cells with the agonist would occur outside the luminometer. Eight seconds is a typical time needed for the movement of the plates at the reading position inside the luminometer. Integrated values were plotted against the logarithm of the

concentration of the agonist. With coelenterazine h, values at high agonist concentrations are inferior to values obtained at a medium agonist concentration, rendering it impossible to draw a correct sigmoidal dose-response curve. This is due to the fact that, for higher agonist concentrations, the kinetics of the signal are rapid (see Figure 5) and most of the light is emitted during the first 8 seconds. In contrast, with coelenterazine i, the dose-response still has a typical sigmoidal shape, as, due to the delay of the signal, only a negligible part of the signal occurs during the 8 first seconds.

Comparison was made between the efficiency of coelenterazine i and the one of other coelenterazines, more precisely coelenterazine h, coelenterazine fcp and coelenterazine ip, for their use in aequorin cell-based assays and results are presented in Figure 6. More precisely, Figure 6 is a graphical analysis of the delay in the luminescent signal obtained with coelenterazine i, in comparison with some other kinds of coelenterazines (h, fcp and ip). Cells were treated as described in Figure 5 and their response to Digitonin (entry of extracellular calcium, open symbols) or their response to serotonin (5HT, closed symbols), an agonist of a receptor expressed by the cells, was analysed. The time at which the maximum of the emission of light occured was calculated with the « Winglow » software provided with the « microlumat » luminometer (Berthold) and is represented here as a function of the concentration of serotonin (left part of the figure) or for a saturating concentration of digitonin (10 µM, right part of the figure). As shown by Figure 6, the use of coelenterazine i delayed the maximum of the signal by 5 to 12 seconds - depending on the concentration of the agonist used -compared with other coelenterazines.

Example 2

A CHO cell line expressing the histamine H1 receptor and apoaequorin was established. Cells were treated as described in Example 1 and were dispensed after dilution (50 µl/well, corresponding to 25 000 cells) into 50 µl of solutions of known agonists for this receptor. The emitted light was recorded during 30 s for each well. Dose-response curves obtained for histamine, an agonist of this receptor, are shown in Figure 8. In other words, Figure 8 represents the same experiment and analysis as described in Figure 7, but here cells expressed the histamine H1 receptor instead of a serotonin receptor, and histamine was used as an agonist of this receptor instead of serotonin. Figure 8 shows that again, the use of coelenterazine i instead of coelenterazine h allows the signal that occurs between the mixing of the cells with the agonist and the start of the light measurement to be excluded while keeping good relevance of the recorded data.

Example 3

A CHO cell line expressing the orexin receptor 2, the Gα16 coupling protein and apoaequorin was established. Cells were treated as described in Example 1 and were dispensed after dilution (50 µl/well, corresponding to 25 000 cells) into 50 µl of solutions of known agonists for this receptor. The emitted light was recorded for a 30 second time period for each well. Dose-response curves obtained for orexin B, an agonist of this receptor, are shown in Figure 9. Therefore, Figure 9 represents the same experiment as described in Figures 7 and 8, but here cells expressed a orexin receptor 2 instead of a serotonin receptor or a histamine receptor, and orexin B was used as an agonist of this receptor, instead of

serotonin or histamine. Again, the use of coelenterazine i instead of coelenterazine h allows the signal that occurs between the mixing of the cells with the agonist and the start of the light measurement to be excluded while keeping good relevance of the recorded data.

Example 4

This example illustrates the use of coelenterazine i with a luminometer where there is a delay between injection of the cells into the solution of the agonist to be tested (or vice-versa) and the beginning of the recording of the emitted light. In this example, reference is made to the results presented on Figure 10.

Series of dilutions of agonists of the MCH receptor were dispensed (20µl/well) in a 384 well assay plate.

In parallel, CHO-apoaequorin-MCH-R cells were prepared as described in previous experiments and 5 µM coelenterazine i were added to the cell suspension to reconstitute active aequorin.

The assay plate containing agonists was placed in the « NorthStar » luminometer (PE Biosystems) and cells (20 µl/well) were injected into each well of the assay plate by means of the NorthStar dispensing system. The plate was then carried by the NorthStar device at the reading position (i.e . under the CCD camera) and measurement of the emitted light was performed for 90 seconds. With the NorthStar, delay between dispensing of the cells on the agonists and start of the measurement of the emitted light is about 16 seconds. Values (RLU, Relative Luminescence Units) were plotted against the logarithm of the agonist concentration to yield the sigmoidal dose-response curves shown in Figure 10. Half maximal agonist concentration (EC50) and maximal effect

(TOP) were calculated from these curves. Digitonin was used as a control for the aequorin content of the cells. Several curves, corresponding to the signal recorded on different columns of the assay plate, are shown in Figure 10.

This experiment demonstrates the utility of delaying the aequorin signal by the use of coelenterazine i, as the same experiment, when performed with coelenterazine h instead of coelenterazine i (and without any delay reagent such as BAPTA), gives negative results with the NorthStar device (data not shown). Said negative results can be explained by the fact that, with coelenterazine h, when the NorthStar device starts the measurement of the emitted light, there is at the time point of measurement almost no emission of light from the coelenterazine h treated cells.

Similar results were obtained when a mixture of cells and antagonists (test compound) were injected into a solution of an agonist (reference agonist), for the search of antagonists of receptors.

The same is true for the injection of an agonist into a mixture of cells, for the search of agonists of receptors, or, for the injection of an agonist into a mixture of cells and antagonists, for the search of antagonists of receptors. In the case where antagonists were searched for, said antagonist and cells were added together or in subsequent steps to the luminometer. When added together to the microplate, the antagonist was preincubated in a separate vial with the cells; when added separately to the microplate, the cells were incubated with the antagonist within the microwell before the agonist was added.

Example 5

This example illustrates the use of coelenterazine i for the detection of antagonists in the concept of the present invention. In this example, reference is made to the results presented on Figure 11.

A series of dilutions of mesulergine, an antagonist of the serotonin 2B receptor, were pre-disposed (50µl/well) in a 96 well assay plate.

In parallel, CHO-aequorin-serotonin 2B receptor cells were prepared as described in previous examples and 5 µM coelenterazine i or coelenterazine h were added to the cell suspension to reconstitute active aequorin.

Cells were dispensed on the dilutions of antagonist and incubated for 30 minutes with the antagonists.

Serotonin, a reference agonist, was then dispensed on the mixture of cells and antagonist, by the means of the dispensing equipment of the "Microlumat" luminometer (Berthold), and the emitted light was immediately measured for 60 seconds.

For coelenterazine i treated cells, there was a 10 second delay between injection and the start of the measurement. Values (RLU, Relative Luminescence Units) were plotted against the logarithm of the antagonist concentration to yield the sigmoidal dose-response curves shown in Figure 11. Half maximal agonist concentration (IC50) was calculated from these curves. Digitonin was used as a control of the aequorin content of the cells.

This example illustrates that coelenterazine i can also be used in assays designed to detect antagonists of calcium-coupled receptors. Half maximal inhibitory effect concentrations for antagonists (IC50) were the same when coelenterazine h or coelenterazine i was used (thus

coelenterazine i is a valuable method for such assays). It allows an incorporation of a delay period between the assay setup and the recording of the final signal.

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CLAIMS

1. Screening, diagnostic and/or dosage method of a known or unknown agonist (full agonist or partial agonist) and/or a known or unknown antagonist (neutral antagonist or inverse agonist) for a calcium-coupled receptor, comprising following steps:

(a) incubating one or more cells expressing apoaequorin and said calcium-coupled receptor with "coelenterazine i" having the formula as depicted in Figure 3 or a derivative thereof, in order to reconstitute active aequorin by said cells,

(b) contacting said cell(s) with the agonist and/or antagonist of said receptor ,

(c) measuring light emitted by said cell(s) on a solid support, allowing the screening, diagnosis and/or dosage of said agonist and/or antagonist of said receptor, and,

(d) possibly, recovering said agonist or antagonist of said receptor from the reaction mixture of step (b).

2. The screening, diagnostic and/or dosage method according to claim 1 wherein the derivative of coelenterazine i is a compound similar to coelenterazine i as depicted in Figure 3, wherein the I is substituted by another halogen chosen from the group consisting of F, Cl, Br; or wherein one or more non-functional groups of coelenterazine i are substituted by other non-functional groups.

3. The screening, diagnostic and/or dosage method of a known or unknown agonist or a known or unknown inverse agonist for a calcium-coupled receptor according to

claim 1 or 2, which comprises the following successive steps:

(a) contacting the agonist or the inverse agonist (tested substance) with a solid support,

(b) incubating one or more cell(s) expressing apoaequorin and said calcium-coupled receptor with coelenterazine i as depicted in Figure 3 or a derivative thereof in order to reconstitute active aequorin by said cell(s),

(c) mixing said agonist or inverse agonist with said cell(s) on said solid support,
OR,
transferring said solid support to a luminometer and mixing said agonist or inverse agonist with said cells in said luminometer, said luminometer being equipped with built-in injector(s),

(d) optionally incubating said cells with said tested substance,

(e) optionally transferring said solid support to a luminometer when said solid support was not previously inserted into the luminometer in step (c),

(f) obtaining a measurement of light emitted by said cell(s),

(g) determining from the results of said measurement whether the tested substance is an agonist or an inverse agonist of said receptor, and, possibly determining the affinity of said tested substance for said receptor, and,

(h) possibly recovering said agonist or said inverse agonist of said receptor from the reaction mixture of step (d).

4. The screening, diagnostic and/or dosage method for a known or an unknown antagonist (neutral

antagonist or inverse agonist) according to claim 1 or 2, which comprises the following successive steps:

(a) contacting the antagonist (tested substance) with a solid support,

(b) incubating one or more cell(s) expressing apoaequorin and said calcium-coupled receptor with coelenterazine i as depicted in Figure 3 or a derivative thereof in order to reconstitute active aequorin by said cell(s),

(c) mixing said antagonist (tested substance) with said cell(s) on said solid support,

(d) optionally incubating said cells with said antagonist,

(e) optionally transferring said solid support to a luminometer and obtaining a measurement of emitted light by said cell(s),

(f) adding a known agonist of the receptor to the mixed cell(s) and tested substance to determine whether the tested substance is an antagonist of the receptor,

(g) optionally transferring said solid support to a luminometer and possibly obtaining a second measurement of light emitted by said cell(s),

(h) determining from the results of steps (e) and/or (g) whether the tested substance is an antagonist of said receptor, and, possibly determining the affinity of said tested substance for said receptor, and,

(i) possibly recovering said antagonist of said receptor from the reaction mixture of step (f).

5. The screening, diagnostic and/or dosage method for a known or an unknown antagonist (neutral antagonist or inverse agonist) according to claim 1 or 2, which comprises the following successive steps:

(a) contacting a known agonist of said calcium-coupled receptor with a solid support,

(b) incubating one or more cell(s) expressing apoaequorin and said calcium-coupled receptor with coelenterazine i as depicted in Figure 3 or a derivative thereof in order to reconstitute active aequorin by said cell(s),

(c) mixing said antagonist (tested substance) with said cell(s)in a vial other than the solid support,

(d) optionally incubating said cells with said antagonist,

(e) optionally transferring said device to a luminometer and obtaining a measurement of light emitted by said cell(s),

(f) adding said cell(s) to the agonist on the solid support to determine whether the tested substance is an antagonist of the receptor,

(g) optionally transferring said solid support to a luminometer and possibly obtaining a second measurement of light emitted by said cell(s),

(h) determining from the results of steps (e) and/or (g) whether the tested substance is an antagonist of said receptor, and, possibly determining the affinity of said tested substance for said receptor, and,

(i) possibly recovering said antagonist of said receptor from the reaction mixture of step (f).

6. The screening, diagnostic and/or dosage method for a known or unknown agonist or a known or unknown inverse agonist according to claim 1 or 2, which comprises the following successive steps:

(a) incubating one or more cell(s) expressing apoaequorin and said calcium-coupled receptor with coelenterazine i as depicted in Figure 3 or a

derivative thereof in order to reconstitute active aequorin by said cell(s),

(b) mixing said agonist or inverse agonist (tested substance) with the cell(s) of step (a) in a vial other than the solid support,

(c) contacting the mixture obtained in step (b) with a solid support,

(d) optionally incubating said cells with said tested substance,

(e) optionally transferring said solid support to a luminometer and obtaining a measurement of light emitted by said cell(s),

(f) determining from the results of said measurement whether the tested substance is an agonist (full or partial) or an inverse agonist of said receptor, and, possibly determining the affinity of said tested substance for said receptor, and,

(g) possibly recovering said agonist or said inverse agonist of said receptor from the reaction mixture of step (d).

7. The screening, diagnostic and/or dosage method of a known or an unknown agonist (full or partial) or a known or unknown inverse agonist according to claim 1 or 2, comprising the following successive steps:

(a) contacting one or more cells with a solid support, said cell(s) expressing apoaequorin and said calcium-coupled receptor, incubating said cells with coelenterazine i as depicted in Figure 3 or a derivative thereof in order to reconstitute active aequorin by said cell(s),

(b) adding to said solid support the agonist or the inverse agonist (tested substance) for said calcium-coupled receptor,

OR,

transferring said solid support to a luminometer and mixing said agonist or inverse agonist with said cells in said luminometer, said luminometer being equipped with built-in injector(s),

(c) optionally incubating said cells with said tested substance,

(d) optionally transferring said solid support to a luminometer when said solid support was not previously inserted in the luminometer in step (b),

(e) obtaining a measurement of light emitted by said cell(s),

(f) determining from the results of said measurement whether the tested substance is an agonist or an inverse agonist of said receptor, and, possibly determining the affinity of said tested substance for said receptor, and,

(g) possibly recovering said agonist or said inverse agonist of said receptor from the reaction mixture of step (c).

8. The screening, diagnostic and/or dosage method of known or an unknown antagonists (neutral antagonist or inverse agonist) for a calcium-coupled receptor according to claim 1 or 2, comprising the following successive steps:

(a) contacting one or more cells with a solid support, said cell(s) expressing apoaequorin and said calcium-coupled receptor, incubating said cells with coelenterazine i as depicted in Figure 3 or a derivative thereof in order to reconstitute active aequorin by said cell(s),

(b) adding to said solid support the antagonist (tested substance) for said calcium-coupled receptor.

OR,

transferring said solid support to a luminometer and mixing said antagonist with said cells in said luminometer, said luminometer being equipped with built-in injector(s), and obtaining a measurement of an emitted light by said cell(s),

(c) optionally incubating said cells with said antagonist,

(d) possibly transferring said solid support at step (c) to a luminometer and obtaining a measurement of light emitted by said cell(s),

OR

keeping said solid support outside the luminometer,

(e) adding a known agonist of the receptor to the mixed cell(s) and the tested substance to determine whether the tested substance is an antagonist of the receptor,

(f) optionally transferring said solid support to a luminometer if not inserted at step (d)

(g) possibly obtaining a second measurement of emitted light by said cell(s),

(h) determining from the results of steps (d) and/or (g) whether the tested substance is an antagonist of said receptor, and, possibly determining the affinity of said tested substance for said receptor, and,

(i) possibly recovering said antagonist of said receptor from the reaction mixture of step (e).

9. The screening, diagnostic and/or dosage method of a known or an unknown antagonist (neutral antagonist or inverse agonist) for a calcium-coupled receptor according to claim 1 or 2, comprising the following successive steps:

(a) preparing one or more cells in a separate vial, said cell(s) expressing apoaequorin and said

calcium-coupled receptor, incubating said cells with coelenterazine i as depicted in Figure 3 or a derivative thereof in order to reconstitute active aequorin by said cell(s),

(b) adding to said cells the antagonist (tested substance) for said calcium-coupled receptor,

(c) transferring the cells of step (b) to a solid support,

(d) optionally incubating said cells with said antagonist before or after the transfer of said mixture to said solid support,

(e) possibly transferring said solid support to a luminometer, and obtaining a measurement of light emitted by said cell(s),

OR,

keeping the solid support outside the luminometer

(f) adding an agonist of the receptor to the mixed cell(s) and the tested substance to determine if the tested substance is an antagonist of the receptor,

(g) transferring said solid support to a luminometer if not inserted at step (e) and possibly obtaining a second measurement of light emitted by said cell(s),

(h) determining from the results of steps (e) and/or (g) whether the tested substance is an antagonist of said receptor, and, possibly determining the affinity of said tested substance for said receptor, and,

(i) possibly recovering said antagonist of said receptor from the reaction mixture of step (f).

10. The screening, diagnostic and/or dosage method for a known or a unknown antagonist (neutral

antagonist or inverse agonist) according to claim 1 or 2, which comprises the following successive steps:

(a) contacting said antagonist (tested substance) with a known agonist for said receptor with a solid support,

(b) incubating one or more cell(s) expressing apoaequorin and said calcium-coupled receptor with coelenterazine i as depicted in Figure 3 or a derivative thereof in order to reconstitute active aequorin by said cell(s),

(c) mixing said antagonist/agonist mixture with said cell(s) on said solid support,

(d) optionally incubating said cells with said tested substance,

(e) transferring said solid support to a luminometer and obtaining a measurement of light emitted by said cell(s),

(f) determining from the results of said measurement whether the tested substance is an antagonist of said receptor, and,

(g) possibly recovering said antagonist of said receptor from the reaction mixture of step (d).

11. The screening, diagnostic and/or dosage method of a known or an unknown antagonist (neutral antagonist or an inverse agonist) according to claim 1 or 2, comprising the following successive steps:

(a) contacting one or more cells with a solid support, said cell(s) expressing apoaequorin and said calcium-coupled receptor, incubating said cells with coelenterazine i as depicted in Figure 3 or a derivative thereof in order to reconstitute active aequorin by said cell(s),

(b) adding to said solid support a known agonist and the tested substance which is an antagonist

(neutral antagonist or an inverse agonist) for said calcium-coupled receptor,

(c) optionally incubating said cells with said antagonist/agonist mixture,

(d) transferring said solid support to a luminometer,

(e) obtaining a measurement of light emitted by said cell(s),

(f) determining from the results of said measurement whether the tested substance is an antagonist of said receptor, and, possibly determining the affinity of said tested substance for said receptor, and,

(g) possibly recovering said antagonist of said receptor from the reaction mixture of step (c).

12. The screening, diagnostic and/or dosage method according to any one of the claims 1 to 11, characterised in that the cell expresses apoaequorin in the cytoplasm or in the mitochondria or in any other part of the cell.

13. The screening, diagnostic and/or dosage method according to any one of the claims 1 to 12, wherein the cell expressing the calcium-coupled receptor also expresses proteins (endogenous or overexpressed) ensuring a coupling of said receptor (endogenous or overexpressed) to a calcium pathway.

14. The screening, diagnostic and/or dosage method according to any one of the claims 1 to 13, wherein said calcium-coupled receptor is a G-protein-coupled receptor (GPCR).

15. The screening, diagnostic and/or dosage method according to claim 13, wherein said protein is selected from the group consisting of natural Gα16 protein, chimeric G-protein resulting from a fusion between

two different G-proteins or phospholipase Cβ2 protein or any other coupling protein or chemical.

16. The screening, diagnostic and/or dosage method according to any of the claims 1 to 15, characterised in that the measurement of the emitted light is obtained with one or more luminometer(s) equipped with several dispensers and measurement heads.

17. The screening, diagnostic and/or dosage method according to any of the claims 1 to 15, characterised in that the measurement of the emitted light is obtained with one or more luminometer(s) wherein no built-in dispensers are present.

18. The screening, diagnostic and/or dosage method according to claim 17, wherein said luminometer is chosen from the group consisting of the NorthStar (PE-Biosystems) Microlumat (Berthold), FLIPR (Molecular Devices) and ImageTrack (Packard).

19. The screening, diagnostic and/or dosage method according to any of the claims 1 to 18, characterised in that the solid support is a microtiter plate.

20. The screening, diagnostic and/or dosage method according to any of the claims 1 to 19, whereby said method is a high throughput method.

21. A screening, diagnostic and/or dosage device intended for the screening, diagnostic and/or dosage method according to any one of the claims 1 to 20, comprising the following elements :

- a microtiter plate, preferably a 96-well microtiter plate,
- a medium containing cell(s) expressing apoaequorin and a calcium-coupled receptor,

- a medium containing coelenterazine i or a derivative thereof, and
- means for detecting light emitted by said cell(s).

22. The device according to claim 21, comprising means for the automatic performance of the successive steps of the diagnostic and/or dosage method according to any one of the claims 1 to 20.

23. A kit for high throughput screening, diagnostic and/or dosage method of a known or unknown agonist and/or a known or an unknown antagonist of a calcium-coupled receptor using a luminometer with a several dispensers and measurement heads or using a luminometer without built-in dispensers; comprising coelentrazine i as depicted in Figure 3 or a derivative thereof.

24. Unknown agonist or unknown antagonist of a receptor identifiable by the method according to any of the claims 1 to 20.

25. Use of an unknown agonist and/or an unknown antagonist of a receptor according to claim 24, in the preparation of a medicament for treating diseases influenced by said receptor.

26. Use of a method according to any of the claims 1 to 20 for the screening, diagnosis and/or dosage of a known or unknown agonist (full agonist or partial agonist) and/or a known or unknown antagonist (neutral antagonist or inverse agonist) for a membrane-linked receptor.

27. Use of a method according to claim 26, wherein said membrane-linked receptor is a G-protein-coupled receptor.

28. A method to screen for, to diagnose and/or dose a known or unknown agonist (full agonist or partial agonist) and/or a known or unknown antagonist

(neutral antagonist or inverse agonist) for a membrane-linked receptor according to any of the claims 1 to 20 capable of modifying the cellular metabolism in a subject.

29. A method according to claim 28 whereby said subject is of human, animal or plant origin.

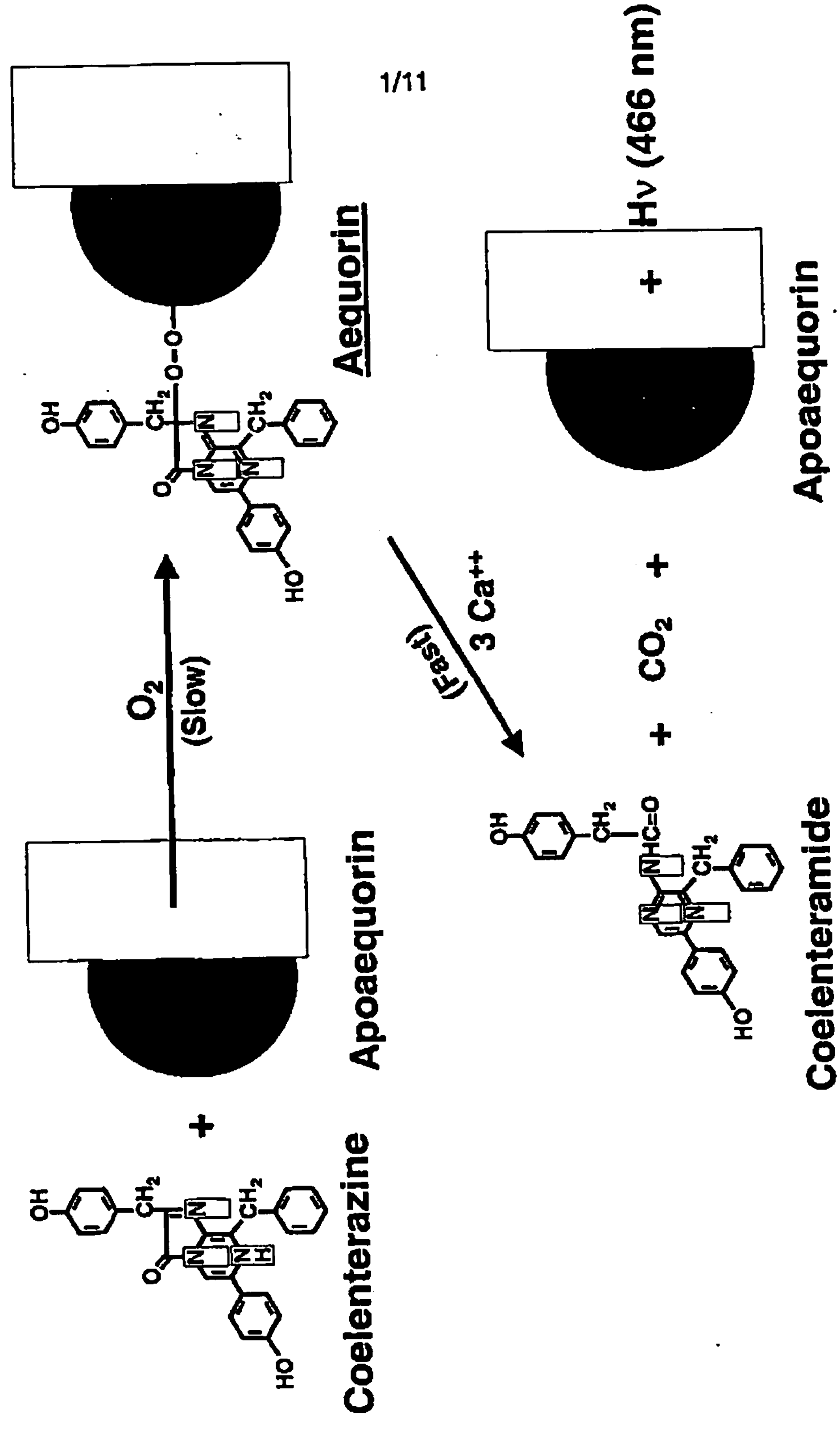

Coelenterazine
+
Apoaequorin
O_2
(Slow)
Aequorin
(Fast)
3 Ca^{++}
Coelenteramide
+
CO_2
+
Apoaequorin
+
Hν (466 nm)

Figure 1

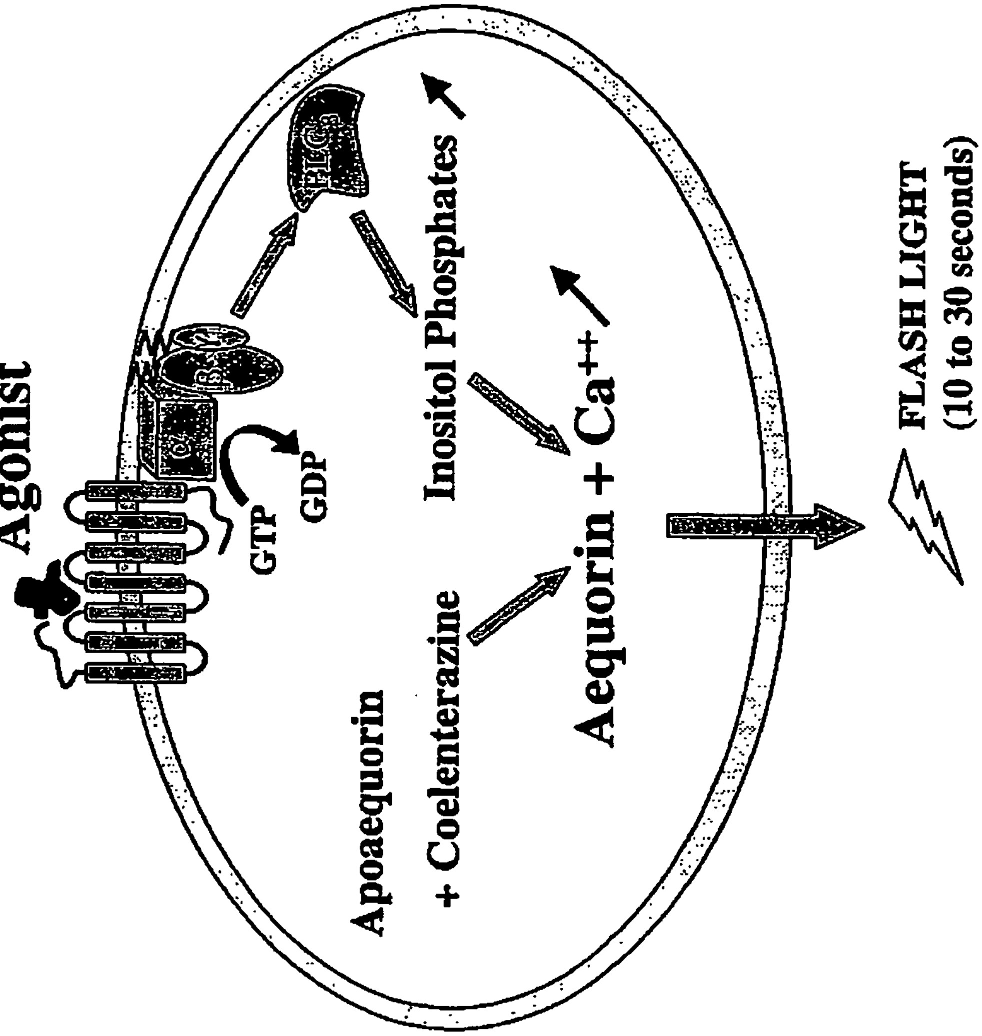
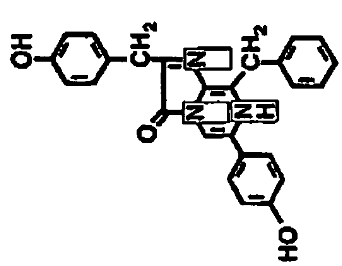
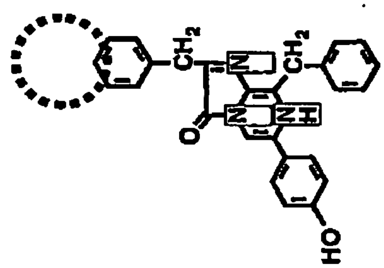

Agonist
GTP
GDP
Apoaequorin
+ Coelenterazine
Inositol Phosphates
Aequorin + Ca++
FLASH LIGHT
(10 to 30 seconds)

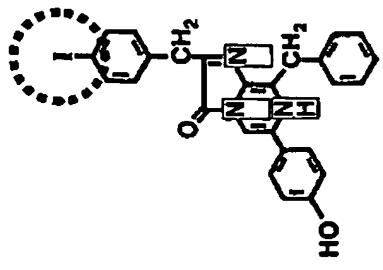
Figure 2



Native coelenterazine



Coelenterazine h



Coelenterazine i

Figure 3

coelenterazine	Emission Maximum (nm)	Relative luminescence capacity	Relative intensity	Half-Rise time (s)
native	465	1.00	1.00	0.4 – 0.8
cp	442	0.95	15	0.15 – 0.3
f	473	0.80	18	0.4 – 0.8
fcp	452	0.57	135	0.4 – 0.8
h	464	0.82	10	**0.4 – 0.8**
hcp	444	0.67	190	0.15 – 0.3
i	476	0.70	0.03	**8**
ip	441	0.54	47	1
n	467	0.26	0.01	5

Figure 4

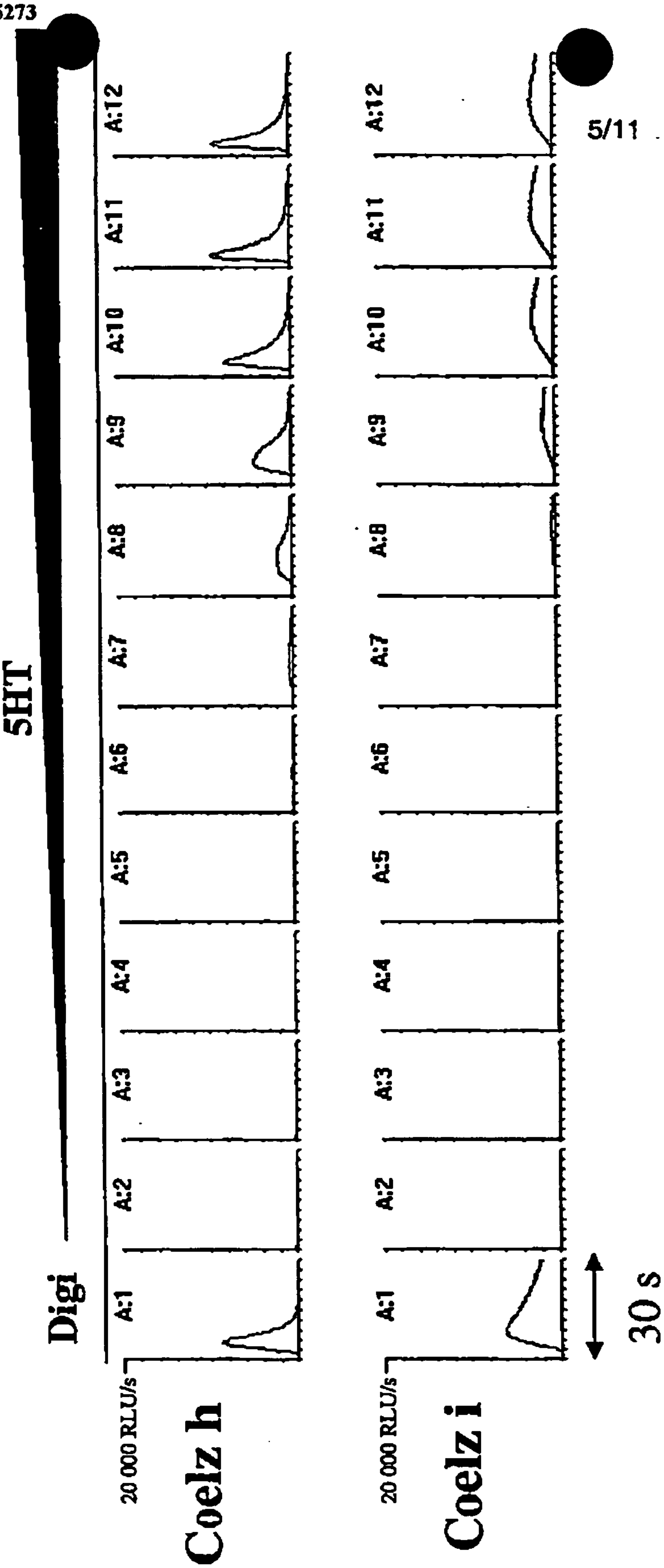

5HT
Digi
A:1
A:2
A:3
A:4
A:5
A:6
A:7
A:8
A:9
A:10
A:11
A:12
20 000 RLU/s
Coelz h
Coelz i
30 s

Figure 5

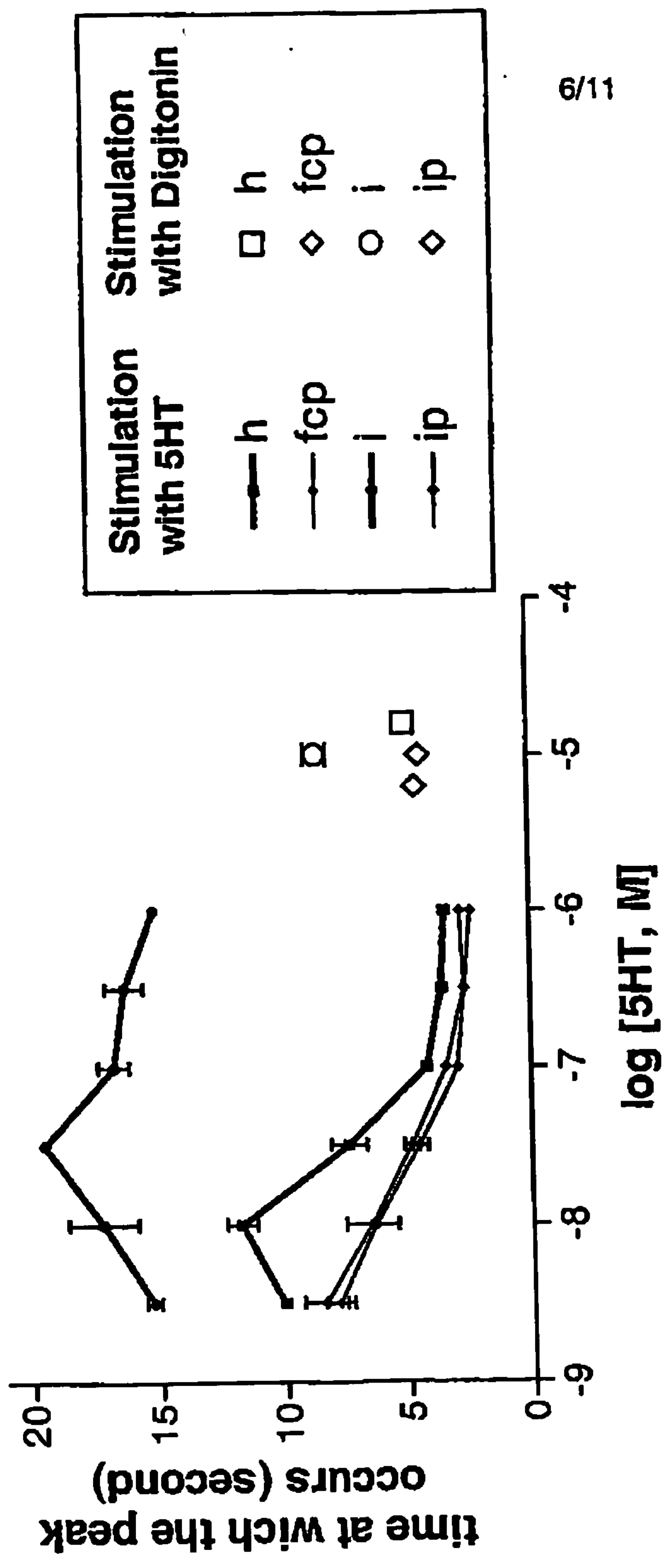

Stimulation with 5HT
h
fcp
i
ip
Stimulation with Digitonin
h
fcp
i
ip
time at wich the peak occurs (second)
0
5
10
15
20
-9
-8
-7
-6
-5
-4
log [5HT, M]

Figure 6

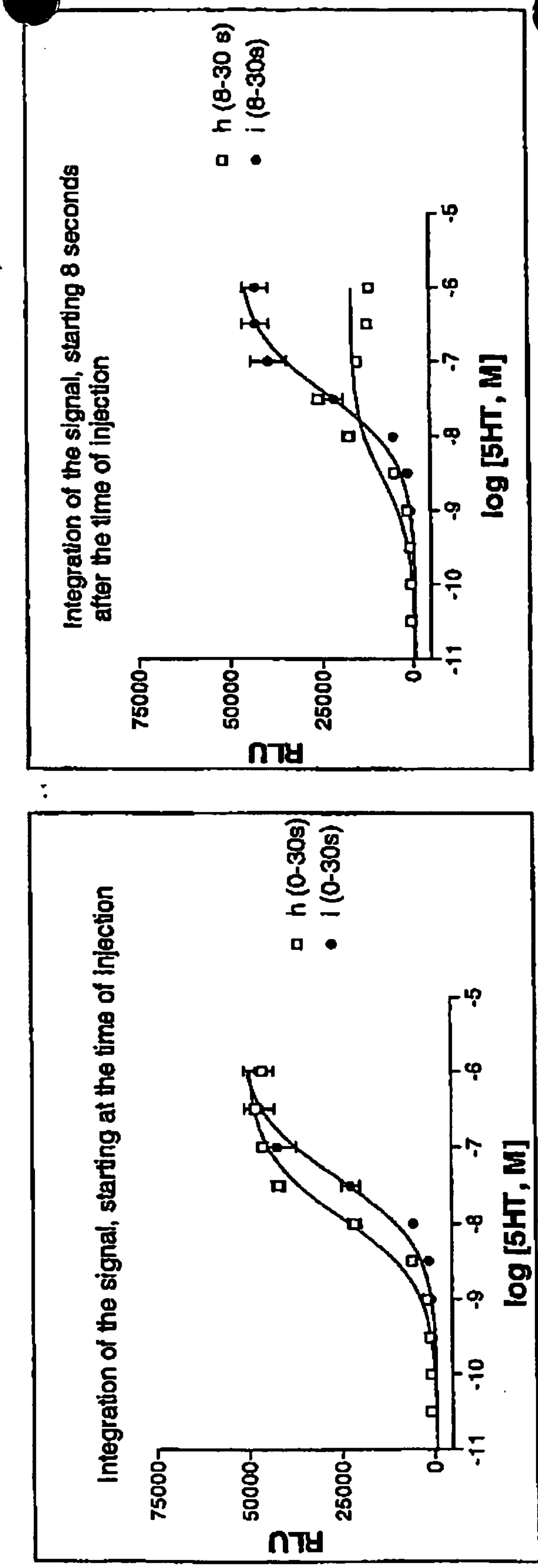

Integration of the signal, starting 8 seconds after the time of injection
h (8-30 s)
i (8-30s)
RLU
75000
50000
25000
0
-11
-10
-9
-8
-7
-6
-5
log [5HT, M]
Integration of the signal, starting at the time of injection
h (0-30s)
i (0-30s)
RLU
75000
50000
25000
0
-11
-10
-9
-8
-7
-6
-5
log [5HT, M]

Figure 7

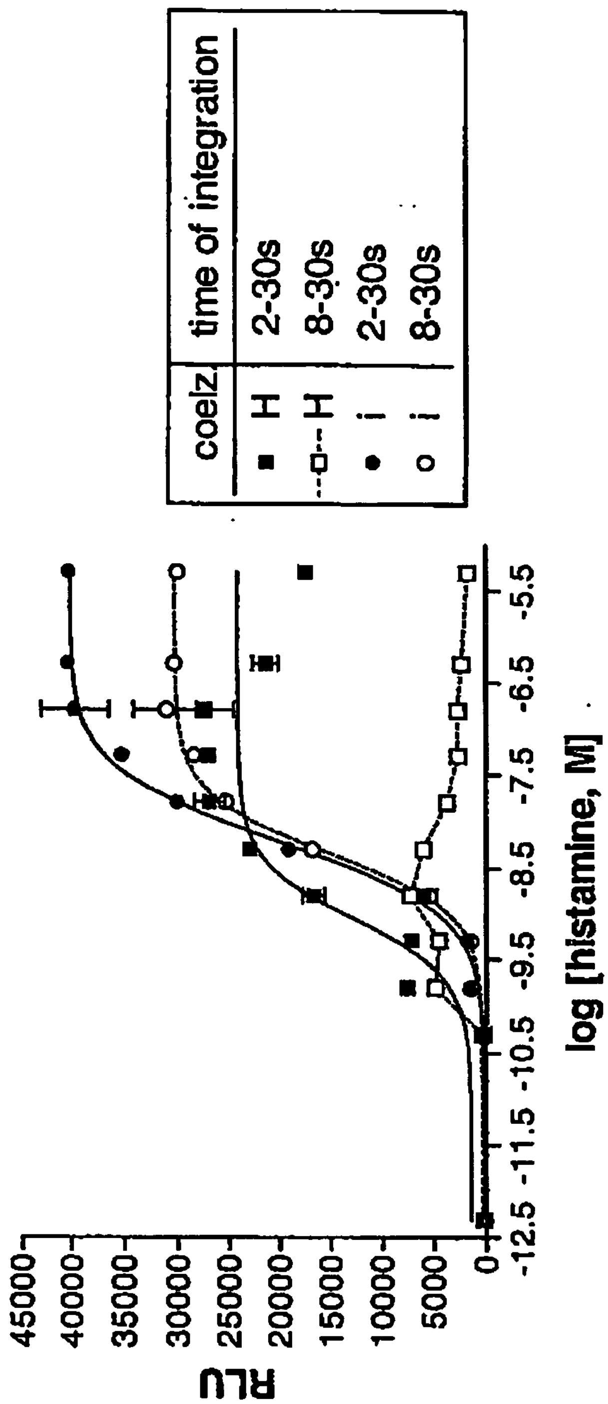

coelz
time of integration
H
2-30s
H
8-30s
2-30s
8-30s
RLU
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
-12.5
-11.5
-10.5
-9.5
-8.5
-7.5
-6.5
-5.5
log [histamine, M]

Figure 8

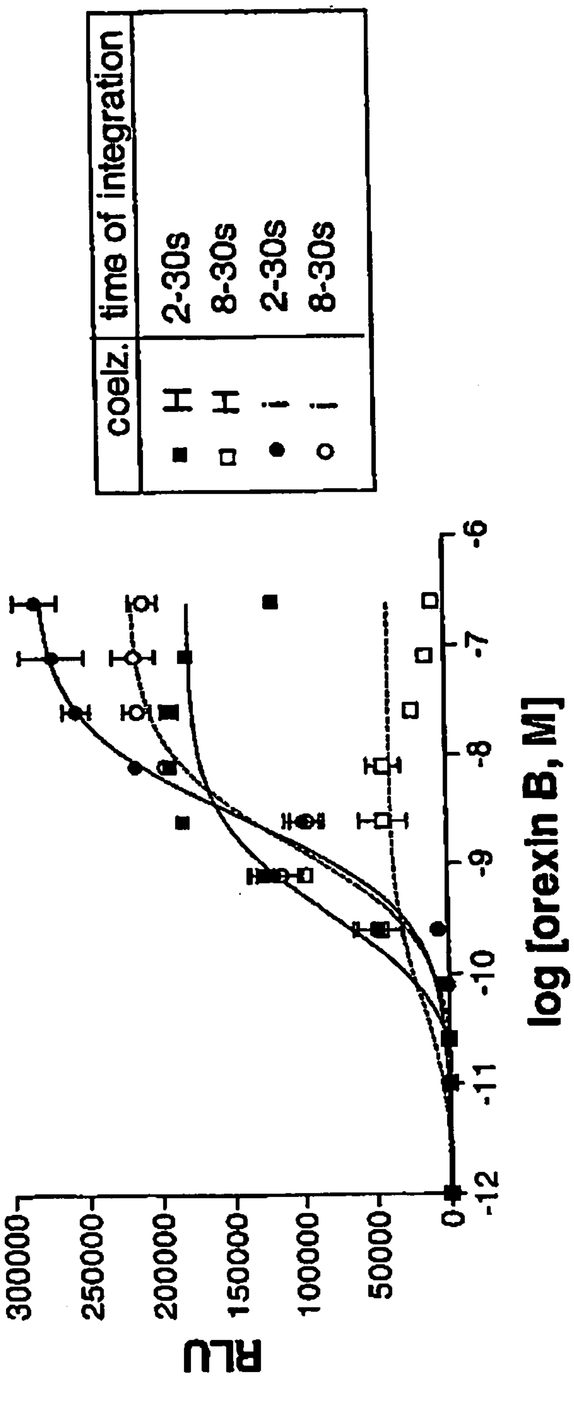
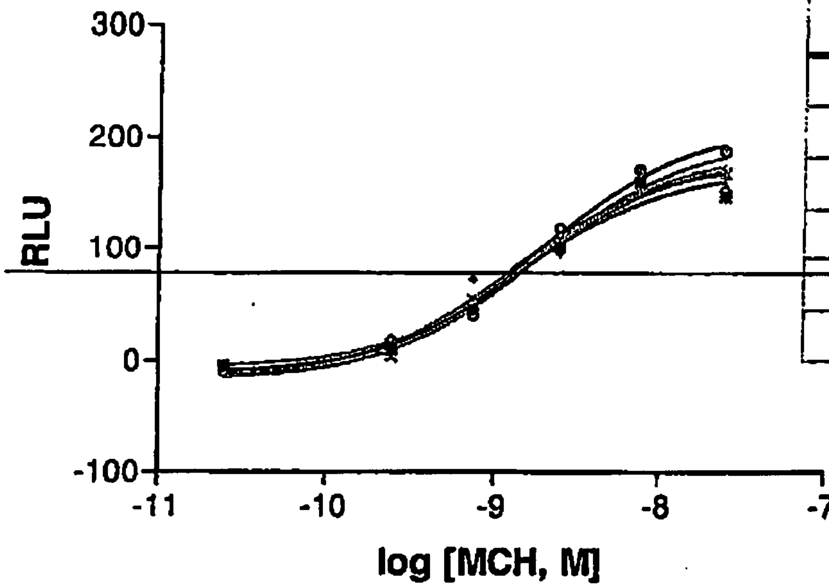

coelz.
time of integration
H 2-30s
H 8-30s
i 2-30s
i 8-30s
RLU
300000
250000
200000
150000
100000
50000
0
-12
-11
-10
-9
-8
-7
-6
log [orexin B, M]

Figure 9

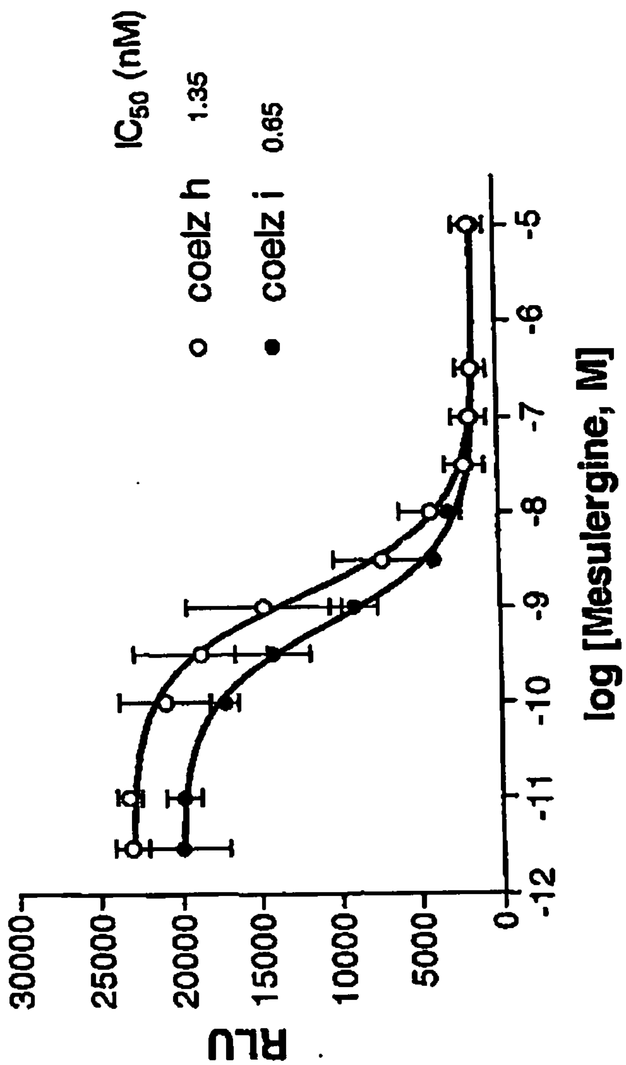


	EC50 (nM)	TOP (RLU)	Moy Digi (RLU)	%TOP/ DIGI
▪ 13	1.47	172.5	138	125.23
' 14	1.59	183.4	169	108.68
× 15	1.63	187.5	155	121.16
◇ 16	1.29	179.2	148	120.88
• 17	1.93	197.2	163	120.98
○ 18	1.89	209.4	179	116.82

Figure 10

5

Figure 11


IC50 (nM)
coelz h 1.35
coelz i 0.65
RLU
30000
25000
20000
15000
10000
5000
0
-12
-11
-10
-9
-8
-7
-6
-5
log [Mesulergine, M]

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(71) Applicant *(for all designated States except US)*: EUROSCREEN S.A. [BE/BE]; Route de Lennik 802, B-1070 Bruxelles (BE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: DUPRIEZ, Vincent [BE/BE]; Avenue de Roodebeek 229, B-1030 Bruxelles (BE). PARMENTIER, Marc [BE/BE]; Laarheidestraat 160, B-1650 Beersel (BE).

(74) Agents: DE CLERCQ, Ann et al.; De Clercq, Brants & Partners, E. Gevaertdreef 10 a, B-9830 Sint-Martens-Latem (BE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) Date of publication of the international search report: 28 August 2003

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/075273 A3

(54) Title: SCREENING, DIAGNOSTIC AND/OR DOSAGE METHOD AND DEVICE OF AN AGONIST AND/OR ANTAGONIST FOR A CALCIUM-COUPLED RECEPTOR

(57) Abstract: The present invention relates to a screening, diagnostic and/or dosage method of a known or unknown agonist (full agonist or partial agonist) and/or a known or unknown antagonist (neutral antagonist or inverse agonist) for a calcium-coupled receptor, comprising following steps:incubating one or more cells expressing apoaequorin and said calcium-coupled receptor with "coelenterazine i" having the formula as depicted in Figure 3 or a derivative thereof, in order to reconstitute active aequorin by said cells; contacting said cell(s) with the agonist and/or antagonist of said receptor on a solid support, and,measuring light emitted by said cell(s), allowing the screening, diagnosis and/or dosage of said agonist and/or antagonist of said receptor, and, possibly, recovering said agonist or antagonist of said receptor from the reaction mixture. The present invention also relates to a corresponding device allowing the performance of said method and to the agonist and/or antagonist of said calcium-coupled receptor identified by said method and device.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/03121

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/58 G01N33/84 G01N33/566 G01N33/94 C12Q1/25

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, PAJ, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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-/--

[X] Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
18 March 2003	26/03/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Vanmontfort, D

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/03121

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SHIMOMURA O ET AL: "SEMI-SYNTHETIC AEQUORINS WITH IMPROVED SENSITIVITY TO CA2+ IONS" BIOCHEMICAL JOURNAL, PORTLAND PRESS, LONDON, GB, vol. 261, no. 3, 1 August 1989 (1989-08-01), pages 913-920, XP001007915 ISSN: 0264-6021 cited in the application abstract; table 1 page 916, column 1, line 60 -column 2, line 33 page 918, column 1, line 48 - line 52	1-23, 26-29
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X	US 6 194 406 B1 (KUDLACZ ELIZABETH M ET AL) 27 February 2001 (2001-02-27) abstract	24,25
A	SHIMOMURA O ET AL: "THE RELATIVE RATE OF AEQUORIN REGENERATION FROM APOAEQUORIN AND COELENTERAZINE ANALOGUES" BIOCHEMICAL JOURNAL, PORTLAND PRESS, LONDON, GB, vol. 296, no. 3, 15 December 1993 (1993-12-15), pages 549-551, XP001011593 ISSN: 0264-6021 the whole document	1-29
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 02/03121

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			NZ	325581	A	27-03-2000
			PT	874843	T	31-12-2002
			SI	874843	T1	31-12-2002
			WO	9722604	A1	26-06-1997